Plant Physiol. (1973) 52, 416–421

Terpene Biosynthesis in Cell-free Extracts and Excised Shoots from Wedgwood Iris[1]

Received for publication February 7, 1973

GEORGE L. STABY,[2] WESLEY P. HACKETT,[3] AND A. A. DE HERTOGH
Department of Horticulture, Michigan State University, East Lansing, Michigan 48823

ABSTRACT

Excised shoots and cell-free extracts prepared from Wedgwood iris (*Iris hollandica* Hoog. "Wedgwood") shoots metabolized ^{14}C-labeled mevalonic acid (MVA). By using cell-free extracts, the ^{14}C from MVA-1-^{14}C was recovered as $^{14}CO_2$, while that from MVA-2-^{14}C was recovered as neutral terpenes, acid-hydrolyzable terpenes, or $^{14}CO_2$. Also, under optimal incubation conditions, 12.8 nanomoles R-MVA-2-^{14}C was incorporated into neutral terpenes per milligram fresh weight per hour. In contrast, excised shoots incorporated only 0.58 nanomoles R-MVA-2-^{14}C per mg fresh weight per hour. Labeled products identified from the cell-free system were squalene, farnesol, geranylgeraniol, and compounds that are converted to farnesol and geranylgeraniol after alkaline hydrolysis. Squalene and a 4,4-dimethylsterol were identified as products from excised shoots but not the terpene alcohols or the alkaline-hydrolyzable compounds.

Cell-free systems for studying terpene biosynthesis have been prepared from many plant species (2–4, 10, 19, 20). Such systems are generally rapid in incorporating labeled precursors into terpene compounds but may not reflect *in vivo* biosynthetic relationships. The products of such a system must be identified and compared with those of an intact system to evaluate the validity of using a cell-free system for studying the relationship of a biosynthetic pathway to plant growth and development. The present study reports characteristics of a cell-free system for biosynthesis of terpenes from MVA-2-^{14}C using extracts of Wedgwood iris shoots and compares the products of the cell-free system with those formed by the incorporation of MVA-2-^{14}C by excised shoots.

MATERIALS AND METHODS

Materials. Current season, temperature (30 C) retarded (15) *Iris hollandica* Hoog "Wedgwood" bulbs (10 cm) were received at 2-week intervals from United Bulb Co., Mount Clemens, Michigan. Upon arrival, the bulbs were placed in dry storage at 9 C for 6 to 8 weeks to promote flower initiation and development (23). Shoots from these low temperature-treated bulbs were used both as a source of enzymes for cell-free studies and as a source of shoot tissue for *in vivo* studies.

All chemicals used were reagent grade. Mevalonate (MVA)-2-^{14}C (dibenzoylethylenediamine salt), MVA-1-^{14}C (lactone), and acetate-1-^{14}C were obtained from New England Nuclear Corp. The specific radioactivities were 1.54 and 8.6 mc/mmole for MVA-1-^{14}C and acetate-1-^{14}C, respectively. The specific radioactivity for MVA-2-^{14}C varied between 4.7 and 6.5 mc/mmole, depending on the sample lot.

Radioactive Monitoring. Measurements of radioactivity were made in a Packard Tri-Carb scintillation spectrometer Model 3310. Each sample was dissolved in 15.0 ml of scintillation fluor which consisted of 100 mg of POPOP and 4 g of PPO in 1 liter of toluene. All samples were counted for 10 min, and the counting efficiency was 80%. The radioactivity on thin layer chromatographic plates was scanned with a Vanguard Autoscanner 880. $^{14}CO_2$ was trapped using a radiorespirometer (27). The trapping solution consisted of 10 ml of absolute ethanol-ethanolamine (1:1). Aliquots of the trapping solution were added to scintillation fluor and counted.

Thin Layer Chromatography. Silica Gel G chromatograms were made on 5 × 20 cm glass plates to a thickness of 0.25 mm. All plates were dried for 1 hr at 100 C prior to use. Plates used for the reversed-phase technique for separation of terpene alcohols were immersed for 1 min in a 5% solution of paraffin oil in petroleum ether (boiling range 30–60 C) and the solvent allowed to evaporate for 15 min with the plate in the horizontal position. The various solvents used are noted in the text and the distance of the solvent run was 15 cm. Standards were detected by spraying plates with a 0.5% solution of iodine in chloroform.

Gas-Liquid Chromatography. Samples were run on a Packard gas chromatograph 7300 equipped with a hydrogen flame ionizing detector using a 6 ft glass column packed with 2% OV-1 on 100/120 Chromosorb W at 230 C with a flow rate of 40 ml/min using N_2 as the carrier gas.

Preparation and Incubation of Cell-free Extracts. Shoot tissue from cold-treated bulbs was homogenized at 0 C for 2 min at top speed in a Lourdes mixer using a 0.1 M tris buffer (1:3 w/v) at pH 7.7 containing 50 μg/ml of chloramphenicol, filtered through cheesecloth, and centrifuged for 30 min at 15,000*g*. The shoots used were 3 to 4 cm in length, about 0.5 cm in diameter, and consisted of floral primordia, a short stem axis (basal plate) and leaves which made up approximately 90% of the fresh weight. The resulting supernatant was used as the source of enzymes for incubation with radioactive substrates and necessary cofactors. Estimates of trichloroacetic acid precipitable protein in the supernatant were by the Lowry (18) procedure.

The standard incubation medium consisted of 2.5 ml of the

[1] Michigan Agricultural Experiment Station Journal Article No. 6086.

[2] Present address: Department of Horticulture, The Ohio State University, Columbus, Ohio 43210.

[3] Present address: Department of Environmental Horticulture, University of California, Davis, Calif. 95616.

supernatant solution, 1.0 ml of tris buffer, pH 7.7, 10 μmoles each of $MnCl_2$, $MgCl_2$, and ATP, and 17 nmoles of MVA-2-^{14}C in a total volume of 4 ml. The mixture was incubated for 1 hr at 30 C in a water bath shaker at 150 rpm.

Incubation of Excised Shoots With Radioactive Mevalonate. Shoots from cold-treated bulbs were excised in such a manner that the leaves and floral primordia remained attached to the basal plate tissue which was 2 mm thick. Ten shoots were floated in 10 ml of 0.1 M tris buffer, pH 7.7, containing 50 μg/ml of chloramphenicol and 0.17 μmole of MVA-2-^{14}C in 125-ml Erlenmyer flasks and incubated for 6 hr at 30 C in a water bath shaker at 150 rpm.

Extraction of Radioactive Reaction Products. Three methods were used. In experiments to assay quantitatively incorporation of MVA-2-^{14}C into neutral terpenes, the reaction was stopped by adding 4 ml of absolute ethanol and 0.5 ml of 50% KOH, and the mixture was slowly brought to a boil. After cooling, neutral lipids were extracted twice with 10 ml of hexane. The hexane was evaporated to dryness *in vacuo* at 60 C, and the residue was suspended in scintillation fluor for counting. The incubation and extraction procedures described above are hereafter referred to as the standard method of assay and extraction. In some experiments, hexane-extracted incubation mixtures were treated with 0.8 N HCl at 37 C for 1 hr and re-extracted with hexane to determine incorporation of radioactivity in the acid-hydrolyzable terpene fraction.

In experiments to determine the nature of the radioactive products biosynthesized from MVA-2-^{14}C, incubations of cell-free extracts were terminated by adding 4 ml of acetone. The precipitate was separated by centrifugation and extracted twice with a total of 4 ml of acetone. The combined acetone extracts were added to the supernatants of the incubation mixtures and enough water added to make them 1:1, acetone-water. This mixture was extracted twice with a total volume of 15 ml of benzene. The combined organic extracts were evaporated with a stream of nitrogen to a small volume, which was transferred quantitatively to the origin of a thin layer plate.

At the termination of the experiments using excised shoots, the tissue was rinsed with distilled water to remove unreacted MVA from the surface, killed by placing in 30 ml of acetone and homogenized for 5 min in the acetone in a Lourdes mixer. Acetone homogenates were transferred to 250-ml Erlenmyer flasks with 25 ml of distilled water and extracted twice with a total of 50 ml of benzene. The organic extracts were reduced to 15 ml under vacuum at 37 C and subsequently evaporated under a stream of N_2 to a small volume which was quantitatively transferred to the origin of a thin layer plate or suspended in scintillation fluor.

RESULTS

General Characteristics of Cell-free System. Both MVA-1-^{14}C and MVA-2-^{14}C were metabolized by iris extracts with radioactivity being recovered only as $^{14}CO_2$ when MVA-1-^{14}C was the substrate and mainly as neutral terpenes when MVA-2-^{14}C was used (Fig. 1). There was, however, a small amount of $^{14}CO_2$ released when MVA-2-^{14}C was used as the substrate (Fig. 2). This release of $^{14}CO_2$ from MVA-2-^{14}C was not detectable until after 30 to 60 min of incubation and using crude cell-free extracts the amount released was approximately 8% of that released when MVA-1-^{14}C was the substrate.

The release of $^{14}CO_2$ from MVA-1-^{14}C was immediate, while there was a lag of approximately 10 min before ^{14}C-neutral terpenes were detected as a result of incubation with MVA-2-^{14}C (Fig. 1). The total amount of radioactivity detected as $^{14}CO_2$

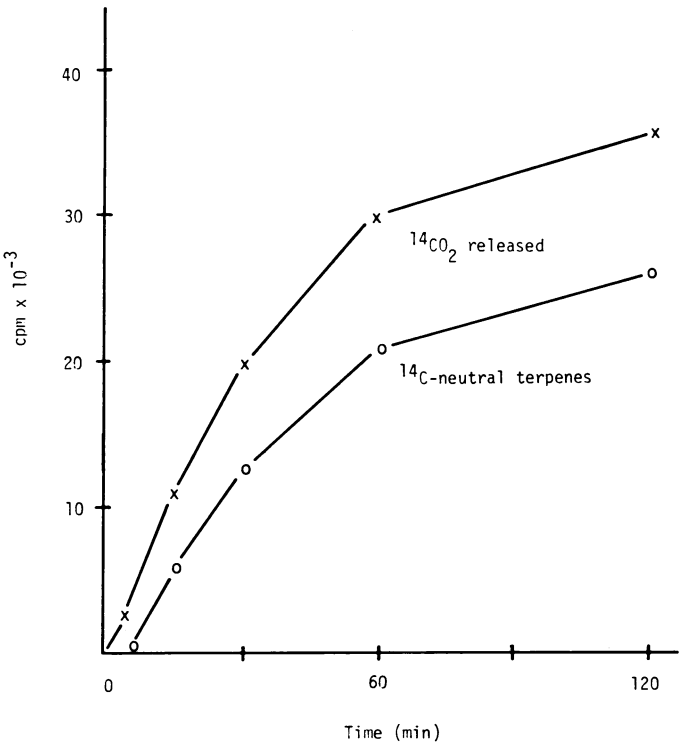

FIG. 1. Effect of time on the incorporation of MVA-2-^{14}C into neutral terpenes and on the release of $^{14}CO_2$ from MVA-1-^{14}C by the cell-free system from iris. Standard extraction and incubation used as described in "Materials and Methods" except for incubation time and substrate indicated.

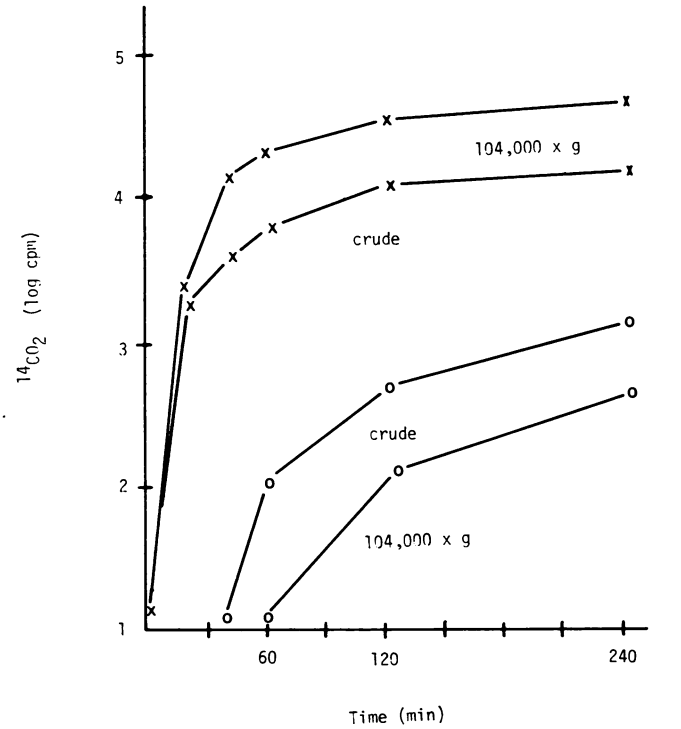

FIG. 2. Time course release of $^{14}CO_2$ from MVA-1-^{14}C (×——×) and MVA-2-^{14}C (○——○) using crude and 104,000*g* supernatant of the cell-free system. Standard extraction and incubation used as described in "Materials and Methods" except incubation time, centrifugation, and substrate as indicated.

Table I. *Influence of Buffers on Incorporation of MVA-2-^{14}C into Neutral and Acid-hydrolyzable Terpene Fractions using Cell-free Systems*

Standard incubation and extraction are as described under "Materials and Methods," except for acid hydrolysis and variation in buffer and pH.

Buffer	MVA-2-^{14}C Incorporated	
	Neutral fraction	Acid-hydrolyzable fraction
0.1 M	*nmoles*	
Tris, pH 7.7	3.26	1.18
Tris, pH 7.4	3.11	0.95
Phosphate, pH 7.4	2.82	2.36

Table II. *Association of ^{14}C-Terpene with Protein as Influenced by Various Treatments of the Cell-free System*

Standard assays were run, except that KOH was added to only one-half of the assays after incubation. Protein was precipitated by treating assay mixtures with trichloroacetic acid. The trichloroacetic acid precipitate and supernatant were extracted separately after ± alkaline hydrolysis.

Treatment	^{14}C-Terpene
	cpm
Nonsaponified (ethyl ether extract)	15,370
Nonsaponified (trichloroacetic acid protein extract)	59,270
Total	74,640
Saponified (ethyl ether extract)	46,690
Saponified (trichloroacetic acid protein extract)	33,090
Total	79,780
Nonsaponified (butanol wash of trichloroacetic acid protein)	67,600
Nonsaponified (trichloroacetic acid protein after butanol wash)	0

Table III. *Radioactivity in Benzene-Acetone Extracts from Excised Iris Shoots Associated with Digitonin Precipitable Sterols following Incubation with MVA-2-^{14}C*

Fraction	Radioactivity	^{14}C-Sterols
	cpm	*%*
Benzene-acetone extract	17,108	
Sterol (+3% ethanolic KOH)	1,702	9.9
Sterol (−3% ethanolic KOH)	1,785	10.4
Sterol (minus digitonin)	85	0.5

from MVA-1-^{14}C was higher than the radioactivity found in the neutral terpenes from MVA-2-^{14}C. This can be explained by the fact that not all of the ^{14}C-products from MVA-2-^{14}C are neutral terpenes (Table I). The metabolism of both substrates was linear for 1 hr. Acetate-1-^{14}C in the presence or absence of NADPH or NADH or both was not substantially incorporated into neutral terpenes.

Data not presented showed that: (*a*) linear increases in the concentrations of R-MVA-2-^{14}C resulted in linear increases in ^{14}C-neutral terpenes biosynthesized. The highest concentration tested (11.7 μM) resulted in an incorporation rate equivalent to 0.3 nmole of R-MVA-2-^{14}C/hr·mg protein. (*b*) Enzyme concentrations of up to 10.5 mg of protein per assay, as determined by trichloroacetic acid precipitable protein, resulted in linear increases in incorporation of MVA-2-^{14}C into neutral terpenes. (*c*) The optimum temperature for incorporation of MVA-2-^{14}C into neutral terpenes was 30 C with little or no activity at 10 C or below and at 50 C and above. (*d*) Anaerobic (N_2) incubation conditions had little or no effect on the incorporation of MVA-2-^{14}C into neutral terpenes and the amount of $^{14}CO_2$ released from MVA-1-^{14}C. (*e*) There was an absolute requirement for ATP for conversion of MVA-2-^{14}C to neutral terpenes and NAD, NADP, and NADPH inhibited slightly incorporation of mevalonate into neutral terpenes. (*f*) Optimal manganese ion concentrations for incorporation of MVA-2-^{14}C into neutral terpenes was between 1.25 mM and 2.5 mM. Magnesium ion stimulated an approximately linear increase in incorporation up to the highest concentration used (5.0 mM).

Optimal incorporation of MVA-2-^{14}C into neutral terpenes occurred at pH 7.7 using 0.1 M tris buffer and at pH 7.4 using 0.1 M phosphate (KH_2PO_4-K_2HPO_4) buffer. The maximum activity was slightly higher with tris than with phosphate buffer. Table I shows that there is a greater proportion of radioactivity in the acid hydrolyzable fraction with phosphate buffer at its optimal pH than with tris at its optimum.

The active enzymes for incorporation of MVA-2-^{14}C into neutral terpenes remained in the soluble fraction after centrifugation at 104,000*g* for 60 min (data not presented). Results presented in Figure 2 show the influence of the crude and 104,000*g* supernatant fraction on the release of $^{14}CO_2$ from MVA-1-^{14}C. With MVA-1-^{14}C as the substrate, more $^{14}CO_2$ was released from the supernatant fraction than from the crude fraction. When MVA-2-^{14}C was used as the substrate, the results were reversed in that release of $^{14}CO_2$ was greater with the crude fraction than with the high spin supernatant. Also the lag time before detection of $^{14}CO_2$ from MVA-2-^{14}C was decreased using the crude extract.

Products of the Cell-free System. Approximately 25% of the radioactivity incorporated into neutral terpenes co-chromatographed with squalene using TLC (five solvent systems) and gas-liquid chromatography (data not reported).

Standard assays were run, except that KOH was added to only one-half of the assays after incubation. Protein was precipitated by treating assay mixtures with trichloroacetic acid. The trichloroacetic acid precipitate and supernatant were extracted separately after ± alkaline hydrolysis. Table II shows that a substantial amount of the radioactive products were associated with trichloroacetic acid precipitable protein and that treatment with KOH caused a partial release of the ^{14}C-products associated with protein to the fraction extractable with ethyl ether. Extraction of the protein-^{14}C-products complex with butanol resulted in all of the radioactivity being transferred to the butanol or acetone. Alkaline hydrolysis of the reaction products also gave rise to more polar substances. Results in Figure 3A show a nonsaponified acetone-benzene extract of the cell-free system. Squalene co-chromatographs with the peak at R_F 0.77. The elution of the radioactive peak at R_F 0.5 with benzene-acetone (1:1) and treatment of the residue with 3% ethanolic KOH for 2 hr at 75 C resulted in a more polar ^{14}C-product at R_F 0.06. (Fig. 3B). Eluting the peak at R_F 0.06 in benzene-acetone (1:1) and rechromatographed using reverse-phase TLC resulted in a scan shown in Figure 3C. Two main ^{14}C-products co-chromatographed with geranylgeraniol and farnesol. Five other solvent systems using TLC techniques also indicated the presence of these two prenols. These data suggest that geranylgeraniol and farnesol, or their pyrophosphate derivatives, are in association with an unknown substance and that when associated, they are less polar than the freed alcohols after alkaline hydrolysis. Based on these results, extraction procedures for experiments on the nature of the

products were modified as described under "Materials and Methods."

Digitonin precipitates were prepared from acetone-benzene extracts of the cell-free system using the procedure of Sperry and Webb (24). Prior to digitonin precipitation, the extracts were treated with 3% ethanolic KOH for 2 hr at 75 C. These precipitates had little or no radioactivity (data not presented) even when 6.0 mM NAD and NADPH were present in the cell-free incubation medium. This indicated that free or esterified sterols are not products of MVA-2-^{14}C incorporation in the iris cell-free system.

Products of Excised Shoots. Both MVA-1-^{14}C and MVA-2-^{14}C were metabolized by excised iris shoot tissue with radioactivity being recovered only as $^{14}CO_2$ when MVA-1-^{14}C was the substrate and only in neutral terpenes and an acid-hydrolyzable fraction when MVA-2-^{14}C was used. Unlike the cell-free extracts there was no net $^{14}CO_2$ released when MVA-2-^{14}C was used as the substrate. Metabolism of MVA-2-^{14}C was less efficient in excised iris shoots than in cell-free extracts of these shoots. In excised shoots, 0.58 nmole of R-MVA-2-^{14}C was incorporated into neutral terpenes/g fresh weight·hr compared to 12.8 nmoles/g fresh weight·hr in the cell-free system. The addition of ATP or cations had no effect on the incorporation of MVA-2-^{14}C by excised shoots (data not presented).

When acetone-benzene extracts obtained from excised iris shoots which had been incubated with MVA-2-^{14}C were chromatographed using hexane and scanned for radioactivity, results such as those shown in Figure 4A were obtained. The most mobile peak co-chromatographed with squalene. When the chromatogram was redeveloped in benzene, the radioactive scan shown in Figure 4B was obtained. Four peaks of radioactivity were apparent, and the most mobile again was co-chromatographed with squalene. The peak at R_F 0.6 approximated in mobility to the cell-free product peak at R_F 0.5 (Fig. 3A). To determine if this peak could be altered by alkaline hydrolysis as it was in the cell-free system, it was eluted, saponified, re-extracted by the methods described above and re-chromatographed in benzene. Radioactive scans showed that the mobility of the peak had not been altered. This indicates that no or undetectable amounts of ^{14}C-products were present similar to those found at R_F 0.5 (Fig. 3A) in the cell-free system. No further attempts were made to identify this peak.

When digitonin precipitates were prepared (24) from acetone-benzene extracts obtained from iris shoots which had been incubated with MVA-2-^{14}C, the results shown in Table III were obtained. About 10% of the radioactivity was digitonin precipitable with or without KOH hydrolysis. When each peak shown in Figure 4B was eluted individually and precipitated with digitonin, only the precipitate from the peak at R_F 0.2 was radioactive. However, only about 35% of the radioactivity of that peak was precipitable with digitonin. The digitonin complex from this peak was split by heating the precipitate to 60 C in 3 ml of pyridine for 1 hr. When recovered radioactivity was thin layer chromatographed using 4% diethyl ether in methylene chloride or benzene-ethyl acetate (5:1), the radioactive peak was slightly more mobile than lanosterol. Likewise, the radioactivity in the supernatant remaining after digitonin precipitation of the peak from R_F 0.2 in Figure 4B was very slightly more mobile than lanosterol in the same two solvents. This indicates that the digitonin precipitate and the remaining supernatant contained the same or very similar radioactive compounds. Sterols, such as lanosterol, that contain 4,4-dimethyl groupings are known to be only poorly precipitated by digitonin (13).

The radioactive peak was eluted from R_F zone 0 to 0.1 (Fig. 4B) and chromatographed using reversed-phase thin layer

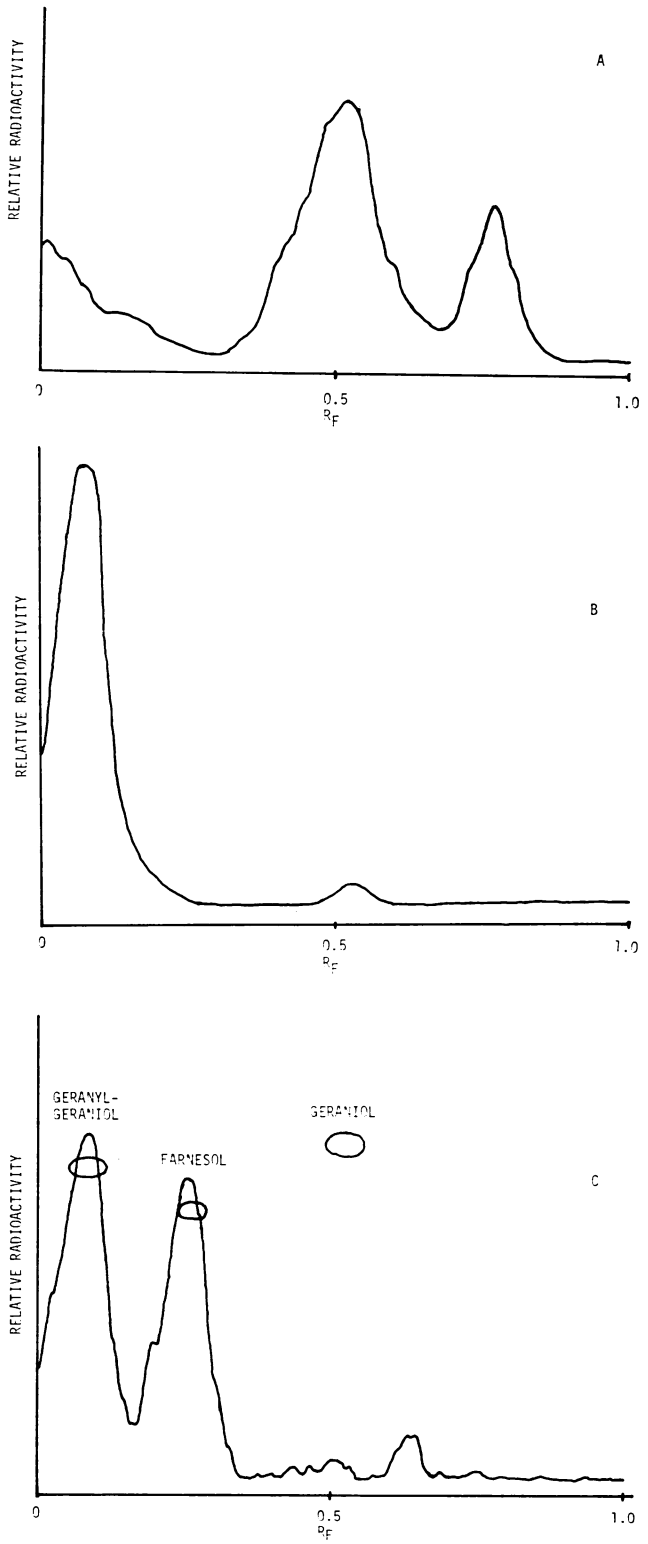

FIG. 3. Thin layer fractionation on silica gel G plates of benzene-acetone-soluble radioactive materials produced from MVA-2-^{14}C by the iris cell-free system. Radioactive scan of: A: plate on which concentrated benzene-acetone extract was chromatographed using benzene; B: plate on which a hexane extract of the KOH-treated eluate from R_F 0.45 to 0.55, Figure 3A was chromatographed using benzene, and C) Plate on which the eluate of the saponification products (R_F 0.05 to 0.1, Fig. 3B) were reversed-phase chromatographed using 70% methanol.

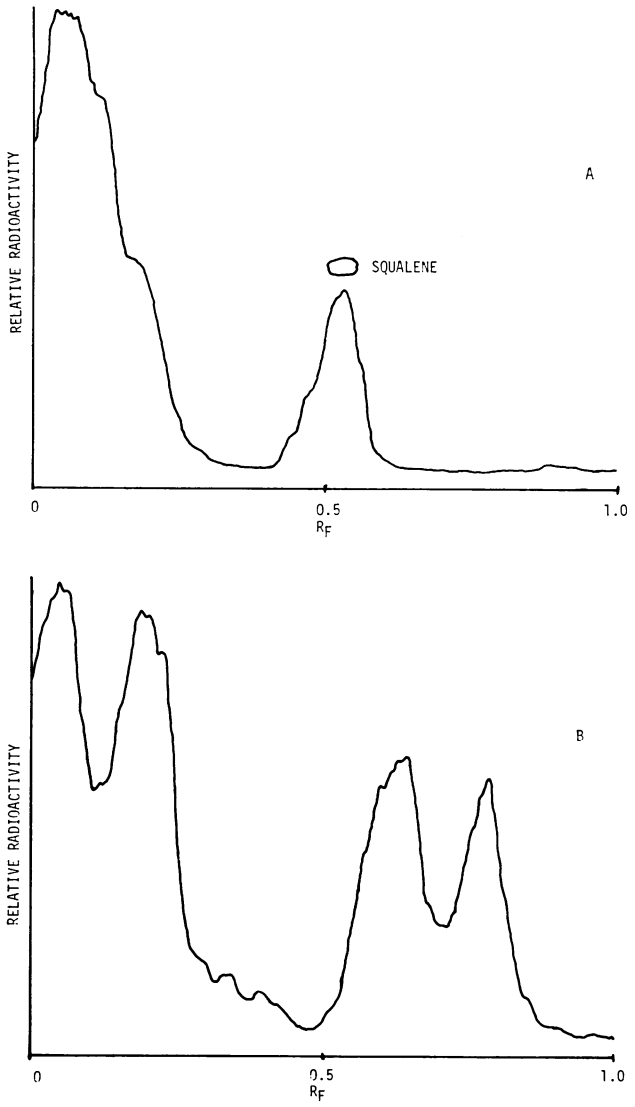

FIG. 4. Thin layer fractionation on silica gel G plates of benzene-actetone-soluble radioactive materials produced from MVA-2-^{14}C by excised iris shoots. Radioactive scan of: A: plate on which concentrated benzene-acetone extract was chromatographed using *n*-hexane and B: same plate redeveloped in benzene.

plates developed in 70% methanol. A radioactive scan of this chromatogram showed two very small peaks, one at R_F 0.1 and another at R_F 0.8, but the majority of the activity was distributed throughout the chromatogram. This indicates that the majority of the activity in R_F zone 0 to 0.1 was not associated with free terpene alcohols which are discreetly separated by this reversed-phase technique.

DISCUSSION

The iris cell-free system incorporated MVA-2-^{14}C into neutral terpenes at a high rate (12.8 nmoles R-MVA-2-^{14}C/mg fresh weight·hr). It has been conclusively shown by others (3, 8, 10, 16) that terpene biosynthesis from MVA proceeds initially to isopentenyl-PP by step-wise addition of 3 ATP to MVA and the loss of the number one carbon of MVA as CO_2 (hereafter referred to as the "normal" biosynthetic pathway). While the intermediates from MVA to isopentenyl-PP were not isolated nor identified in the present study, other data obtained indicated that the biosynthesis of terpenes in iris proceeded in this manner. The absolute requirement for ATP, the stimulation by Mn^{2+} and Mg^{2+}, the kinetics of the $^{14}CO_2$ released from MVA-1-^{14}C (Fig. 1), the absence of a requirement for air and the release of hexane-soluble radioactivity as a result of acid hydrolysis (Table I) and the inhibition of the incorporation of MVA-2-^{14}C by 5.0 mM iodoacetamide (data not reported) all support this conclusion.

The amount of phosphorylated intermediates (acid-hydrolyzable fraction, Table I) accumulated during incubation was increased by use of a phosphate as compared to a tris buffer. This effect is presumably the consequence of inhibition of phosphatase activity. High phosphatase activity has been found in other cell-free terpene biosynthesizing systems (5).

The rapid release of $^{14}CO_2$ from MVA-1-^{14}C and the lack of radioactivity in the neutral terpene fraction when MVA-1-^{14}C was used is expected if the biosynthesis of terpenes in the iris cell-free system is proceeding in the normal manner (22). The release of $^{14}CO_2$ from MVA-2-^{14}C is known to occur in the biosynthesis of sterols (22). However, digitonin precipitation of sterols from the iris cell-free system showed little or no radioactivity associated with sterols. Alternatively, the release of $^{14}CO_2$ from MVA-2-^{14}C incubations may have been a catabolic disposal of certain intermediates. Popjak (21) and Christophe and Popjak (9) reported that ^{14}C terpene alcohols were converted to prenoic acids, especially when microsomes were present and that there was a net loss of ^{14}C (presumably as $^{14}CO_2$) from incubations. These observations could account for the delayed release of $^{14}CO_2$ from MVA-2-^{14}C incubations of iris extracts and the greater $^{14}CO_2$ release from the crude extract incubations as compared to the 104,000*g* supernatant incubations (Fig. 2).

A large percentage of the radioactivity incorporated into neutral terpenes from mevalonate was associated with trichloroacetic acid precipitable proteins (Table II). Similar results have been reported (5, 19). This radioactivity could be in the form of covalently bound intermediates (17) or as a noncovalent association. Since butanol extracted 100% of the radioactivity from the protein, it appears that in the iris system the radioactive products are noncovalently bound to the protein (Table II).

The main products of MVA-2-^{14}C incorporation by cell-free extracts of iris shoots were tentatively identified as squalene, farnesol, geranylgeraniol, and compounds that are converted to farnesol and geranylgeraniol after alkaline hydrolysis. These two alcohols were also tentatively identified in their free form, presumably the result of phosphatase hydrolysis of the corresponding pyrophosphate esters. Products similar to those found *in vitro* in this study as compounds converted to farnesol and geranylgeraniol after alkaline hydrolysis have not been described. However, formate, acetate, propionate, and butyrate esters of these alcohols commonly occur in the essential oils of many plants (26). The presence of such esters would not be observed if incubations were terminated by addition of NaOH or KOH because such treatment would hydrolyze the ester bond to give the free ^{14}C terpene alcohols. Such treatment may account for the lack of reports of these esters as products of cell-free systems.

While it is not known if these compounds that were converted to the two prenols in the iris cell-free system were esters, synthesis of esters of terpene alcohols could probably occur by esterification of the free alcohols in the presence of Mg^{2+}, ATP and CoA (12) if the cell-free extract contains the appropriate kinase and thiokinase enzymes. Magnesium and ATP were included in the incubation mixture of the cell-free extracts but CoA was not. Because of the rather crude nature

of the iris cell-free extracts CoA and the appropriate enzymes might be expected to be present.

Squalene, which was tentatively identified as a product of the iris cell-free system, is a common product from MVA-2-^{14}C in cell-free systems of higher plants (4, 7, 13, 14). Pyridine nucleotides were not required for squalene synthesis in the iris cell-free system; Graebe *et al.* (13) reported similar findings for extracts of *Echinocystis macrocarpa* embryos. These findings may be related to the relatively crude nature of the extracts which could contain sufficient levels of pyridine nucleotides to satisfy the usual requirement. Even though radioactive squalene was tentatively identified, MVA-2-^{14}C was not incorporated into sterols *in vitro*.

Formation of geranylgeraniol from MVA in the cell-free system from iris shoots is of special interest, since geranylgeranyl pyrophosphate is considered a precursor of the gibberellins. However, no evidence for production of kaurene, the first unique precursor of gibberellins, was found. Biosynthesis of kaurene has been reported in cell-free systems from plant fruit and seed tissue but not shoot tissue (2, 13, 14).

Products of MVA-2-^{14}C incorporation by iris shoot tissue differed considerably from those of the iris cell-free extract. Compounds that are converted to farnesol and geranylgeraniol by alkaline hydrolysis were not produced by excised shoots but squalene was produced. At least one 4,4-dimethylsterol of the lanosterol type was biosynthesized in excised shoots, but not in the cell-free system. Using gas-liquid chromatography and mass spectroscopy, Staby and De Hertogh (25) showed that campesterol, stigmasterol, and β-sitosterol are constituents of iris shoots. In the present study, no evidence was found for biosynthesis of these 4,4-dimethylsterols by excised shoots. The 4,4-dimethylsterols are common products of MVA-2-^{14}C incorporation in plants and are thought to be intermediates of phytosterol biosynthesis (11). Perhaps the 6-hr incubation time of the present study was not sufficient for accumulation of radioactive phytosterols which are farther along the biosynthetic pathway than 4,4-dimethylsterols in which radioactivity was detected.

The lack of products capable of being hydrolyzed to farnesol and geranylgeraniol by excised shoots may be due to the fact that free terpene alcohols did not accumulate to any extent in excised shoots. It is commonly believed that terpene alcohols are artifacts (13, 14), although certain terpene alcohols have been shown to be precursors of higher terpenes (1). The lack of net evolution of $^{14}CO_2$ from MVA-2-^{14}C by excised iris shoots is to be expected, since only radioactive 4,4-dimethylsterols were detected. Decarboxylation of triterpene intermediates of phytosterol biosynthesis occurs subsequent to the formation of the 4,4-dimethylsterols (11). Since labeled terpene alcohols did not accumulate when excised shoots were fed MVA-2-^{14}C, evolution of $^{14}CO_2$ from prenoic acids (9, 21) would not be expected.

Although this work demonstrates the biosynthesis of squalene and 4,4-dimethylsterols in excised iris shoot tissue, there was no evidence of biosynthesis of kaurene, a unique precursor of gibberellins. It is known that kaurene accumulates in *Podocarpus ferrugineus* and *P. spicatus* leaves (6) and in some cell-free systems from fruits and seeds (2, 13, 14).

LITERATURE CITED

1. Aller, Van, R. T. and V. R. Nes. 1968. The phosphorylation of geraniol in germinating peas. Phytochemistry 7: 85–88.
2. Anderson, J. D. and T. C. Moore. 1967. Biosynthesis of (−)-kaurene in cell-free extracts of immature pea seeds. Plant Physiol. 42: 1527–1534.
3. Archer, B. L., B. G. Audley, E. G. Cockbain, and G. P. McSweeney. 1963. The biosynthesis of rubber. Incorporation of mevalonate and isopentenyl pyrophosphate into rubber by *Hevea brasiliensis*—latex fractions. Biochem. J. 89: 565–574.
4. Beeler, D. A., D. G. Anderson, and J. W. Porter. 1963. The biosynthesis of squalene from mevalonic acid-2-^{14}C and farnesyl pyrophosphate 4,8,12-^{14}C by carrot and tomato enzymes. Arch. Biochem. Biophys. 102: 26–32.
5. Beytia, E., P. Valenzuela, and O. Cori. 1969. Terpene biosynthesis: formation of nerol, geraniol, and other prenols by an enzyme system from *Pinus radiata* seedlings. Arch. Biochem. Biophys. 129: 346–356.
6. Briggs, L. H., R. W. Cawley, J. A. Loe, and W. I. Taylor. 1950. Diterpenes. III. The diterpenes from *Podocarpus ferrugineus*. J. Chem. Soc. 3: 955–958.
7. Capstack, Jr., E., N. Rosin, G. A. Blandin, and W. R. Nes. 1965. Squalene in *Pisum sativum*. Its cyclization to β-amyrin and labeling pattern. J. Biol. Chem. 240: 3258–3263.
8. Chesterton, C. J. and R. S. O. Kekwick. 1968. Formation of isopentyl monophosphate and pyrophosphate in the latex of *Hevea brasiliensis*. Arch. Biochem. Biophys. 125: 76–85.
9. Christophe, J. and G. Popjak. 1961. Studies on the biosynthesis of cholesterol. XIV. The origin of prenoic acids from alkyl pyrophosphates in liver systems. J. Lipid. Res. 2: 244–257.
10. George-Nascimento, C., E. Beytia, A. R. Aedo, and O. Cori. 1969. Transformation of mevalonic acid into isoprenoid compounds by enzymes from *Pinus radiata*. Activation of the overall reaction by phospholipids. Arch. Biochem. Biophys. 132: 470–476.
11. Goad, L. J. and T. W. Goodwin. 1966. The biosynthesis of sterols in higher plants. Biochem. J. 99: 735–746.
12. Goodman, D. S. 1969. Hydrolysis and formation of cholesterol esters in rat liver. Methods Enzymol. 15: 522–537.
13. Graebe, J. E., D. T. Dennis, C. D. Upper, and C. A. West. 1965. Biosynthesis of gibberellins. I. Biosynthesis of (−)-kaurene, (−)-kaurene-19-ol and transgeranylgeraniol in endosperm nucleus of *Echinocystis macrocarpa* Greene. J. Biol. Chem. 240: 1847–1854.
14. Graebe, J. E. 1968. Biosynthesis of kaurene, squalene, and phytoene from mevalonate-2-^{14}C in a cell-free system from pea fruits. Phytochemistry 7: 2003–2020.
15. Hartsema, A. M. and A. H. Blaauw. 1935. The shifting of periodicity by means of high temperature. Adaption and export to the southern hemisphere II. Proc. Kon. Ned. Akad. Wetensch. 38: 722–734.
16. Heftmann, E. 1970. Steroid Biochemistry. Academic Press, New York.
17. Krishna, G., H. W. Whitlock, D. H. Feldbruegge and J. W. Porter. 1966. Enzymatic conversion of farnesyl pyrophosphate to squalene. Arch. Biochem. Biophys. 114: 200–215.
18. Lowry, O. H., J. N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin reagent. J. Biol. Chem. 193: 265–275.
19. Oster, M. O. and C. A. West. 1968. Biosynthesis of transgeranylgeranyl pyrophosphate in endosperm of *Echinocystis macrocarpa* Greene. Arch. Biochem. Biophys. 127: 112–123.
20. Pollard, C. J., J. Bonner, A. J. Haagen-Smit, and C. C. Nimmo. 1966. Metabolic transformation of mevalonic acid by an enzyme system from peas. Plant Physiol. 41: 66–70.
21. Popjak, G. 1959. Biosynthesis of derivatives of allylic alcohols from 2-^{14}C mevalonate in liver enzyme preparations and their relation to synthesis of squalene. Tetrahedron Lett. 19: 19–28.
22. Richards, J. H. and J. B. Hendrickson. 1964. The Biosynthesis of Steroids, Terpenes and Acetogenins. W. A. Benjamin Inc., New York.
23. Rodrigues Pereira, A. S. 1965. Physiological analysis of flower formation in Wedgwood iris. J. Exp. Bot. 16: 405–410.
24. Sperry, W. M. and M. Webb. 1950. Revision of the Schoenheimer-Sperry method for cholesterol determination. J. Biol. Chem. 187: 97–106.
25. Staby, G. L. and A. A. De Hertogh. 1972. β-Sitosterol, stigmasterol, campesterol and octacosanol in extracts from Wedgewood iris shoots. Hortscience 7: 411–412.
26. Stahl, E. and J. Jork. 1969. *In:* E. Stahl, ed., Thin Layer Chromatography. Springer-Verlag. Berlin.
27. Wang, C. H. 1962. Metabolism studies by radiorespirometry. Atomlight 21: 1–5.